# ENVIRONMENTAL STRESS-TOLERANT PLANTS

## BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a transgenic plant containing a gene in which a DNA encoding a protein that binds to dehydration responsive element (DRE) and regulates the transcription of genes located downstream of DRE is ligated downstream of a stress responsive promoter.

2. Prior Art

In the natural world, plants are living under various environmental stresses such as dehydration, high temperature, low temperature or salt. Unlike animals, plants cannot protect themselves from stresses by moving. Thus, plants have acquired various stress tolerance mechanisms during the courses of their evolution. For example, low temperature tolerant plants (Arabidopsis thaliana, spinach, lettuce, garden pea, barley, beet, etc.) have less unsaturated fatty acid content in their biomembrane lipid than low temperature sensitive plants (maize, rice, pumpkin, cucumber, banana, tomato, etc.). Therefore, even when the former plants are exposed to low temperatures, phase transition is hard to occur in their biomembrane lipid and, thus, low temperature injury does not occur easily.

To date, dehydration, low temperature or salt tolerant lines have been selected and crossed in attempts to artificially create environmental stress tolerant plants. However, a long time is needed for such selection, and the crossing method is only applicable between limited species. Thus, it has been difficult to create a

plant with high environmental stress tolerance.

As biotechnology progressed recently, trials have been made to create dehydration, low temperature or salt tolerant plants by using transgenic technology which introduces into plants a specific, heterologous gene. Those genes which have been used for the creation of environmental stress tolerant plants include synthesis enzyme genes for osmoprotecting substances (mannitol, proline, glycine betaine, etc.) and modification enzyme genes for cell membrane lipid. Specifically, as the mannitol synthesis enzyme gene, Escherichia coli-derived mannitol 1-phosphate dehydrogenase gene [Science 259:508-510 (1993)] was used. As the proline synthesis enzyme gene, bean-derived $\triangle^1$-proline-5-carboxylate synthetase gene [Plant Physiol. 108:1387-1394 (1995)] was used. As the glycine betaine synthesis enzyme gene, bacterium-derived choline dehydrogenase gene [Plant J. 12:1334-1342 (1997)] was used. As the cell membrane lipid modification enzyme gene, Arabidopsis thaliana-derived $\omega$ -3 fatty acid desaturase gene [Plant Physiol. 105:601-605 (1994)] and blue-green alga-derived $\triangle$9 desaturase gene [Nature Biotech. 14:1003-1006 (1996) were used. However, the resultant plants into which these genes were introduced were instable in stress tolerance or low in tolerance level; none of them have been put into practical use to date.

Further, it is reported that a plurality of genes are involved in the acquisition of dehydration, low temperature or salt tolerance in plants [Plant Physiol., 115:327-334 (1997)]. Therefore, a gene encoding a transcription factor capable of activating simultaneously the expression of a plurality of genes involved in the acquisition of stress tolerance has been introduced into plants, yielding plants

with high stress tolerance. However, when a gene which induces the expression of a plurality of genes is introduced into a host plant, the genes are activated at the same time. As a result, the energy of the host plant is directed to production of the products of these genes and intracellular metabolism of such gene products, which often brings about delay in the growth of the host plant or dwarfing of the plant.

## OBJECTS AND SUMMARY OF THE INVENTION

It is an object of the present invention to provide a transgenic plant containing a gene in which a DNA encoding a protein that binds to a stress responsive element and regulates the transcription of genes located downstream of the element is ligated downstream of a stress responsive promoter, the transgenic plant having improved tolerance to environmental stresses (such as dehydration, low temperature and salt) and being free from dwarfing.

Toward the solution of the above problem, the present inventors have cloned a novel transcription factor gene that regulates the expression of genes involved in the acquisition of dehydration, low temperature or salt stress tolerance, and introduced into a plant this novel gene ligated downstream of a stress responsive promoter. As a result, the inventors have succeeded in creating a plant which has remarkably improved tolerance to dehydration, low temperature or salt and which is free from dwarfing. Thus, the present invention has been achieved.

The present invention relates to a transgenic plant containing a gene in which a DNA encoding the following protein (a) or (b) is ligated downstream of a stress responsive promoter:

(a) a protein consisting of the amino acid sequence as shown in SEQ I D NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 or SEQ ID NO: 10;

(b) a protein which consists of the amino acid sequence having deletion, substitution or addition of at least one amino acid in the amino acid sequence as shown in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 or SEQ ID NO: 10 and which regulates the transcription of genes located downstream of a stress responsive element.

Further, the present invention relates to a transgenic plant containing a gene in which the following DNA (c) or (d) is ligated downstream of a stress responsive promoter:

(c) a DNA consisting of the nucleotide sequence as shown in SEQ ID NO : 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7 or SEQ ID NO: 9;

(d) a DNA which hybridizes with the DNA consisting of the nucleotide sequence as shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7 or SEQ ID NO: 9 under stringent conditions and which codes for a protein that regulates the transcription of genes located downstream of a stress responsive element.

Specific examples of the stress include dehydration stress, low temperature stress and salt stress.

As the stress responsive promoter, at least one selected from the group consisting of rd29A gene promoter, rd29B gene promoter, rd17 gene promoter, rd22 gene promoter, DREB1A gene promoter, cor6.6 gene promoter, cor15a gene promoter, erd1 gene promoter and kin1 gene promoter may be given.

This specification includes part or all of the contents as described in the specification and/or drawings of Japanese Patent Application No. 10-292348, which is a priority document of the present application.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagram showing the principle of screening of DREB genes.

Figs. 2A and 2B show the structures of probes used in a gel shift assay on the DRE-binding property of DREB1A and DREB2A proteins and presents electrophoresis photographs showing the results of the gel shift assay.

Figs. 3A and 3B present diagrams showing the transcription activating ability of DREB1A and DREB2A proteins.

Fig. 4 is a diagram showing the structure of a CaMV35S promoter-containing recombinant plasmid to be introduced into a plant.

Fig. 5 presents electrophoresis photographs showing transcription levels of individual genes in DREB1A gene-introduced plants when stress is loaded.

Fig. 6 presents photographs showing the growth of DREB1A gene-introduced plants when freezing stress or dehydration stress is given (morphology of organisms).

Fig. 7 is a diagram showing the structure of a rd29A gene promoter-containing recombinant plasmid to be introduced into a plant.

Figs. 8A and 8B present photographs showing the growth of pBI35S:DREB1A-introduced transgenic plants (morphology of organisms).

Figs. 9A and 9B present photographs showing the growth of pBI29AP:DREB1A-introduced transgenic plants (morphology of organisms).

Fig. 10 presents photographs showing the survival of transgenic plants after stress loading (morphology of organisms).

## DETAILED DESCRIPTION OF THE INVENTION

Hereinbelow, the present invention will be described in detail.

The transgenic plant of the invention is a environmental stress tolerant, transgenic plant created by introducing a gene in which a DNA (called "DREB gene") encoding a transcription factor that binds to a dehydration responsive element (DRE) and activates the transcription of genes located downstream of DRE is ligated downstream of a stress responsive promoter.

The DREB genes used in the invention can be cloned as described below. Of these DREB genes, DRE-binding protein 1A gene is called DREB1A gene; DRE-binding protein 1B gene is called DREB1B gene; DRE-binding protein 1C gene is called DREB1C gene; DRE-binding protein 2A gene is called DREB2A gene; and DRE-binding protein 2B gene is called DREB2B gene.

1. Cloning of DREB Gene

1-1. Preparation of mRNA and a cDNA Library from Arabidopsis thaliana

As a source of mRNA, a part of the plant body of Arabidopsis thaliana such as leaves, stems, roots or flowers, or the plant body as a whole may be used. Alternatively, plant bodies obtained by sowing seeds of Arabidopsis thaliana on a solid medium such as GM medium, MS medium or #3 medium and growing the resultant seedlings aseptically may be used. The mRNA level of DREB1A gene in Arabidopsis thaliana plants increases when they are exposed to low temperature stress (e.g. 10 to -4℃). On the other hand, the mRNA level of DREB2A gene increases when plants are exposed to salt stress (e.g. 150-250 mM NaCl) or dehydration stress (e.g. dehydrated state). Therefore, Arabidopsis thaliana plants which have been exposed to such stress may also be used.

mRNA is prepared, for example, by exposing Arabidopsis thaliana plants grown on GM medium to the dehydration stress, low temperature stress or salt stress mentioned above and then freezing them with liquid nitrogen. Subsequently, conventional techniques for mRNA preparation may be used. For example, the frozen plant are ground in a mortar. From the resultant ground material, a crude RNA fraction is extracted by the glyoxal method, the guanidine thiocyanate-cesium chloride method, the lithium chloride-urea method, the proteinase K-deoxyribonuclease method or the like. From this crude RNA fraction, poly(A)$^+$ RNA (mRNA) can be obtained by the affinity column method using oligo dT-cellulose or poly U-Sepharose carried on Sepharose 2B or by the batch method. The resultant mRNA may further be fractionated by sucrose gradient centrifugation or the like.

Single-stranded cDNA is synthesized using the thus obtained mRNA as a template; this synthesis is performed using a commercial kit (e.g. ZAP-cDNA Synthesis Kit: Stratagene), oligo(dT)$_{20}$ and a reverse transcriptase. Then, double-stranded cDNA is synthesized from the resultant single-stranded cDNA. An appropriate adaptor such as EcoRI-NotI-BamHI adaptor is added to the resultant double-stranded cDNA, which is then ligated downstream of a transcriptional activation domain (such as GAL4 activation domain) in a plasmid (such as pAD-GAL4 plasmid: Stratagene) containing such a domain to thereby prepare a cDNA library.

1-2. A Host to Be Used in the Cloning of DREB Gene

DREB gene can be cloned, for example, by one hybrid screening method using yeast. Screening by this method may be performed using a commercial kit (e.g. Matchmaker One Hybrid System: Clontech).

In the cloning of DREB gene using the above-mentioned kit, first, it is necessary to ligate a DNA fragment comprising DRE sequences to which a protein encoded by DREB gene (i.e. DREB protein) binds to both plasmids pHISi-1 and pLacZi contained in the kit. Then, the resultant plasmids are transformed into the yeast contained in the kit (Saccharomayces cerevisiae YM4271) to thereby prepare a host yeast for cloning.

The host yeast for cloning can biosynthesize histidine by the action of HIS3 protein which is expressed leakily by HIS3 minimum promoter. Thus, usually, this yeast can grow in the absence of histidine. However, since the promoter used for the expression of the gene encoding HIS3 protein is a minimum promoter which can only maintain the minimum transcription level, HIS3 protein produced in cells is extremely small in quantity. Therefore, when the host yeast is cultured in the presence of 3-AT (3-aminotriazole) that is a competitive inhibitor against HIS3 protein, the function of HIS3 protein in cells is inhibited by 3-AT in a concentration dependent manner. When the concentration of 3-AT exceeds a specific level, HIS3 protein in cells becomes unable to function and, as a result, the host yeast becomes unable to grow in the absence of histidine. Similarly, lacZ gene is also located downstream of CYC1 minimum promoter. Thus, $\beta$-galactosidase is produced only in extremely small quantity in the yeast cells. Therefore, when the host yeast is plated on an Xgal containing plate, colonies appearing thereon do not have such Xgal degrading ability that turns the colonies into blue as a whole. However, when a transcription factor that binds to DRE sequences located upstream of HIS3 and lacZ genes and activate the transcription thereof is expressed in the host yeast, the yeast

becomes viable in the presence of a sufficient amount of 3-AT and, at the same time, Xgal is degraded to turn the colonies into blue.

As used herein, the term "dehydration responsive element (DRE)" refers to a cis-acting DNA domain consisting of a 9 bp conserved sequence 5'-TACCGACAT-3' located upstream of those genes which are expressed upon exposure to dehydration stress, low temperature stress, etc.

A DNA fragment comprising DRE can be obtained by amplifying the promoter region of rd29A gene (from -215 to -145 based on the translation initiation site of the gene) by polymerase chain reaction (PCR), rd29 gene being one of dehydration tolerance genes [Kazuko Yamaguchi-Shinozaki and Kazuo Shinozaki, The Plant Cell 6:251-264 (1994)]. As a template DNA which can be used in this PCR, genomic DNA from Arabidopsis thaliana is given. As a sense primer, 5'-aagcttaagcttacatcagtttgaaagaaa-3' (SEQ ID NO: 11) may be used. As an antisense primer, 5'-aagcttaagcttgctttttggaactcatgtc-3' (SEQ ID NO: 12) may be used. Other primers may also be used in the present invention.

1-3. Cloning of DREB1A Gene and DREB2A Gene

DREB1A gene and DREB2A gene can be obtained by transforming the cDNA library obtained in subsection 1-1 above into the host obtained in subsection 1-2 above by the lithium acetate method or the like, plating the resultant transformant on LB medium plate or the like containing Xgal (5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside) and 3-AT (3-aminotriazole), culturing the transformant, selecting blue colonies appearing on the plate and isolating the plasmids therefrom.

Briefly, a positive clone containing DREB1A gene or DREB2A gene

contains a fusion gene composed of a DNA region coding for GAL4 activation domain (GAL4 AD) and a DNA region coding for a DRE-binding protein, and expresses a fusion protein (hybrid protein) composed of the DRE-binding protein and GAL4 activation domain under the control of alcohol dehydrogenase promoter. Subsequently, the expressed fusion protein binds, through the DRE-binding protein moiety, to DRE located upstream of a reporter gene. Then, GAL4 activation domain activates the transcription of lacZ gene and HIS3 gene. As a result, the positive clone produces remarkable amounts of HIS3 protein and β-galactosidase. Thus, because of the action of the HIS3 protein produced, the positive clone can biosynthesize histidine even in the presence of 3-AT. Therefore, the clone becomes viable in the presence of 3-AT and, at the same time, the Xgal in the medium is degraded by the β-galactosidase produced to turn the colonies into blue.

Subsequently, such blue colonies are subjected to single cell isolation, and the isolated cells are cultured. Then, plasmid DNA is purified from the cultured cells to thereby obtain DREB1A gene or DREB2A gene.

1-4. Homologues to DREB1A Protein or DREB2A Protein

Organisms may have a plurality of genes with similar nucleotide sequences which are considered to have evolved from a single gene. Proteins encoded by such genes are mutually called homologues. They can be cloned from the relevant gene library using as a probe a part of the gene of which the nucleotide sequence has already been known. In the present invention, genes encoding homologues to DREB1A or DREB2A protein can be cloned from the Arabidopsis thaliana cDNA library using DREB1A cDNA or DREB2A cDNA obtained in subsection 1-3

above as a probe.

1-5. Determination of Nucleotide Sequences

The cDNA portion is cut out from the plasmid obtained in subsection 1-3 or 1-4 above using a restriction enzyme and ligated to an appropriate plasmid such as pSK (Stratagene) for sub-cloning. Then, the entire nucleotide sequence is determined. Sequencing can be performed by conventional methods such as the chemical modification method by Maxam-Gilbert or the dideoxynucleotide chain termination method using M13 phage. Usually, sequencing is carried out with an automated DNA sequencer (e.g. Perkin-Elmer Model 373A DNA Sequencer).

SEQ ID NO: 1 shows the nucleotide sequence of DREB1A gene, and SEQ ID NO: 2 the amino acid sequence of the protein encoded by this gene. SEQ ID NO: 3 shows the nucleotide sequence of DREB2A gene, and SEQ ID NO: 4 the amino acid sequence of the protein encoded by this gene. SEQ ID NO: 5 shows the nucleotide sequence of DREB1B gene, and SEQ ID NO: 6 the amino acid sequence of the protein encoded by this gene. SEQ ID NO: 7 shows the nucleotide sequence of DREB1C gene, and SEQ ID NO: 8 the amino acid sequence of the protein encoded by this gene. SEQ ID NO: 9 shows the nucleotide sequence of DREB2B gene, and SEQ ID NO: 10 the amino acid sequence of the protein encoded by this gene. As long as a protein consisting of one of the above-mentioned amino acid sequences has a function to bind to DRE to thereby activate the transcription of genes located downstream of DRE, the amino acid sequence may have mutation (such as deletion, substitution or addition) in at least one amino acid. A mutated gene coding for the protein having such mutated amino acid sequence may also be used

in the present invention.

For example, at least 1 amino acid, preferably 1 to about 20 amino acids, more preferably 1 to 5 amino acids may be deleted in the amino acid sequence shown in SEQ ID NO: 2, 4, 6, 8 or 10; at least 1 amino acid, preferably 1 to about 20 amino acids, more preferably 1 to 5 amino acids may be added to the amino acid sequence shown in SEQ ID NO: 2, 4, 8 or 10; or at least 1 amino acid, preferably 1 to about 160 amino acids, more preferably 1 to 40 amino acids may be substituted with other amino acid(s) in the amino acid sequence shown in SEQ ID NO: 2, 4, 8 or 10. A gene coding for a protein having such mutated amino acid sequence may be used in the present invention as long as the protein has a function to bind to DRE to thereby activate the transcription of genes located downstream of DRE.

Also, a DNA which can hybridize with the above-mentioned gene under stringent conditions may be used in the present invention as long as the protein encoded by the DNA has a function to bind to DRE to thereby activate the transcription of genes located downstream of DRE. The "stringent conditions" means, for example, those conditions in which formamide concentration is 30-50%, preferably 50%, and temperature is 37-50 ℃, preferably 42℃.

A mutated gene may be prepared by known techniques such as the method of Kunkel, the gapped duplex method or variations thereof using a mutation introducing kit [e.g. Mutant-K (Takara) or Mutant-G (Takara)] or using LA PCR in vitro Mutagenesis Series Kit (Takara).

Once the nucleotide sequence of DREB gene has been determined definitely, the gene can be obtained by chemical synthesis, by PCR using the cDNA or genomic DNA of the gene as a template, or by

hybridization with a DNA fragment having the above nucleotide sequence as a probe.

The recombinant vectors containing DREB1A gene and DREB2A gene, respectively, were introduced into E. coli K-12 strain and deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (1-3, Higashi 1-Chome, Tsukuba City, Ibaraki, Japan) under accession numbers FERM BP-6654 (E. coli containing DREB1A gene) and FERM BP-6655 ( E. coli containing DREB2A gene) on August 11, 1998.

2. Determination of the DRE Binding Ability and Transcription Activating Ability of the Proteins encoded by DREB Genes

2-1. Analysis of the DRE Binding Ability of the Proteins encoded by DREB Genes

The ability of the protein encoded by DREB gene (hereinafter referred to as the "DREB protein") to bind to DRE can be confirmed by performing a gel shift assay [Urao, T. et al., The Plant Cell 5:1529-1539 (1993)] using a fusion protein composed of the above protein and GST. A fusion protein composed of DREB1A protein and GST can be prepared as follows. First, DREB1A gene is ligated downstream of the GST coding region of a plasmid containing GST gene (e.g. pGEX-4T-1 vector: Pharmacia) so that the reading frames of the two genes coincide with each other. The resultant plasmid is transformed into E. coli, which is cultured under conditions that induce synthesis of the fusion protein. The resultant E. coli cells are disrupted by sonication, for example. Cell debris is removed from the disrupted material by centrifugation. Then, the supernatant is purified by

affinity chromatography using a carrier such as glutathione-Sepharose to thereby obtain the fusion protein.

Gel shift assay is a method for examining the interaction between a DNA and a protein. Briefly, a DRE-containing DNA fragment labelled with $^{32}P$ or the like is mixed with the fusion protein described above and incubated. The resultant mixture is electrophoresed. After drying, the gel is autoradiographed to detect those bands which have migrated behind as a result of the binding of the DNA fragment and the protein. In the present invention, the specific binding of DREB1A or DREB2A protein to the DRE sequence can be confirmed by making it clear that the above-mentioned behind band is not detected when a DNA fragment containing a varied DRE sequence is used.

2-2. Analysis of the Transcription Activating Ability of the Proteins Encoded by DREB Genes

The transcription activating ability of the proteins encoded by DREB genes can be analyzed by a trans-activation experiment using a protoplast system from Arabidopsis thaliana. For example, DREB1A cDNA is ligated to pBI221 plasmid (Clontech) containing CaMV35S promoter to construct an effector plasmid. On the other hand, 3 cassettes of the DRE-containing 71 base DNA region obtained in subsection 1-2 above are connected tandemly to prepare a DNA fragment, which is then ligated upstream of TATA promoter located upstream of $\beta$-glucuronidase (GUS) gene in pBI221 plasmid to construct a reporter plasmid. Subsequently, these two plasmids are introduced into protoplasts of Arabidopsis thaliana and then GUS activity is determined. If GUS activity is increased by the simultaneous expression of DREB1A protein, it is understood that DREB1A protein

expressed in the protoplasts is activating the transcription of GUS gene through the DRE sequence.

In the present invention, preparation of protoplasts and introduction of plasmid DNA into the protoplasts may be performed by the method of Abel et al. [Abel, S. et al., Plant J. 5:421-427 (1994)]. In order to minimize experimental errors resulted from the difference in plasmid DNA introduction efficiency by experiment, a plasmid in which luciferase gene is ligated downstream of CaMV35S promoter may be introduced into protoplasts together with the two plasmids described above, and $\beta$ -glucuronidase activity against luciferase activity may be determined. Then, the determined value may be taken as a value indicating the transcription activating ability. $\beta$ -glucuronidase activity can be determined by the method of Jefferson et al. [Jefferson, R.A. et al., EMBO J. 83:8447-8451 (1986)]; and luciferase activity can be determined using PicaGene Luciferase Assay Kit (Toyo Ink).

3. Creation of Transgenic Plants

A transgenic plant having tolerance to environmental stresses, in particular, low temperature stress (including freezing stress), dehydration stress and salt stress, can be created by introducing the gene obtained in section 1 above into a host plant using recombinant techniques. As a method for introducing the gene into a host plant, indirect introduction such as the Agrobacterium infection method, or direct introduction such as the particle gun method, polyethylene glycol method, liposome method, microinjection or the like may be used. When the Agrobacterium infection method is used, a transgenic plant can be created by the following procedures.

3-1. Preparation of a Recombinant Vector to be Introduced into a Plant and Transformation of Agrobacterium

A recombinant vector to be introduced into a plant can be prepared by digesting with an appropriate restriction enzyme a DNA comprising DREB1A, DREB1B, DREB1C, DREB2A or DREB2B gene obtained in section 1 above, ligating an appropriate linker to the resultant DNA if necessary, and inserting the DNA into a cloning vector for plant cells. As the cloning vector, a binary vector type plasmid such as pBI2113Not, pBI2113, pBI101, pBI121, pGA482, pGAH, pBIG; or an intermediate vector type plasmid such as pLGV23Neo, pNCAT, pMON200 may be used.

When a binary vector type plasmid is used, the gene of interest is inserted between the border sequences (LB, RB) of the binary vector. The resultant recombinant vector is amplified in E. coli. The amplified recombinant vector is introduced into Agrobacterium tumefaciens C58, LBA4404, EHA101, C58C1Rif$^R$, EHA105, etc. by freeze-thawing, electroporation or the like. The resultant Agrobacterium tumefaciens is used for the transduction of a plant of interest.

In addition to the method described above, the three-member conjugation method [Nucleic Acids Research, 12:8711 (1984)] may also be used to prepare DREB gene-containing Agrobacterium for use in plant infection. Briefly, an E. coli containing a plasmid comprising the gene of interest, an E. coli containing a helper plasmid (e.g. pRK2013) and an Agrobacterium are mixed and cultured on a medium containing rifampicin and kanamycin. Thus, a zygote Agrobacterium for use in plant infection can be obtained.

Since DREB gene encodes a protein which activates transcription, various genes are activated by the action of the expressed DREB

protein in a DREB gene-introduced plant. This leads to increase in energy consumption and activation of metabolism in the plant. As a result, the growth of the plant itself may be inhibited. As a means to prevent such inhibition, it is considered to ligate a stress responsive promoter upstream of DREB gene so that the DREB gene is expressed only when a stress is loaded. Specific examples of such a promoter include the following ones:

rd29A gene promoter [Yamaguchi-Shinozaki, K. et al., The Plant Cell 6:251-264 (1994)]

rd29B gene promoter [Yamaguchi-Shinozaki, K. et al., The Plant Cell 6:251-264 (1994)]

rd17 gene promoter [Iwasaki, T. et al., Plant Physiol., 115:1287 (1997)]

rd22 gene promoter [Iwasaki, T. et al., Mol. Gen. Genet., 247:391-398 (1995)]

DREB1A gene promoter [Shinwari, Z.K. et al., Biochem. Biophys. Res. Com. 250:161-170 (1988)]

cor6.6 gene promoter [Wang, H. et al., Plant Mol. Biol. 28:619-634 (1995)]

cor15a gene promoter [Baker, S.S. et al., Plant Mol. Biol. 24:701-713 (1994)]

erd1 gene promoter [Nakashima K. et al., Plant J. 12:851-861 (1997)]

kin1 gene promoter [Wang, H. et al., Plant Mol. Biol. 28:605-617 (1995)]

Other promoter may also be used as long as it is known to be stress responsive and to function in plant. These promoters can be

obtained by PCR amplification using primers designed based on a DNA comprising the promoter and using relevant genomic DNA as a template.

If necessary, it is also possible to ligate a terminator which demands termination of transcription downstream of DREB gene. As the terminator, cauliflower mosaic virus-derived terminator or nopaline synthase gene terminater may be used. Other terminator may also be used as long as it is known to function in plant.

If necessary, an intron sequence which enhances the expression of a gene may be located between the promoter sequence and DREB gene. For example, the intron from maize alcohol dehydrogenase (Adh1) [Genes & Development 1:1183-1200 (1987)] may be introduced.

In order to select transformed cells of interest efficiently, it is preferable to use an effective selection marker gene in combination with DREB gene. As the selection marker, one or more genes selected from kanamycin resistance gene (NPTII), hygromycin phosphotransferase gene (htp) which confers resistance to the antibiotic hygromycin on plants, phosphinothricin acetyl transferase gene (bar) which confers resistance to bialaphos and the like.

DREB gene and the selection marker gene may be incorporated together into a single vector. Alternatively, the two genes may be incorporated into separate vectors to prepare two recombinant DNAs.

3-2. Introduction of DREB Gene into a Host Plant

In the present invention, the term "host plant" means any of the following: cultured plant cells, the entire plant of a cultured plant, plant organs (such as leaves, petals, stems, roots, rhizomes, seeds), or plant tissues (such as epidermis, phloem, parenchyma, xylem, vascular bundle). Specific examples of plants which may be used as a

host include Arabidopsis thaliana, tobacco, rice and maize.

DREB gene can be introduced into the above-described host plant by introducing a DREB gene-containing vector into plant sections by the Agrobacterium infection method, particle gun method or polyethylene glycol method. Alternatively, a DREB gene-containing vector may be introduced to protoplasts by electroporation.

If a gene of interest is introduced by the Agrobacterium infection method, a step of infecting a host plant with an Agrobacterium containing a plasmid comprising the gene of interest is necessary. This step can be performed by the vacuum infiltration method [CR Acad. Sci. Paris, Life Science, 316:1194 (1993)]. Briefly, Arabidopsis thaliana is grown in a soil composed of vermiculite and perlite (50:50). The resultant plant is dipped directly in a culture fluid of an Agrobacterium containing a plasmid comprising DREB gene, placed in a desiccator and then sucked with a vacuum pump to 65-70 mmHg. Then, the plant was allowed to stand at room temperature for 5-10 min. The plant pot is transferred to a tray and covered with a wrap to maintain the humidity. The next day, the wrap is removed. The plant is grown in that state to harvest seeds.

Subsequently, in order to select those individuals which have the gene of interest, seeds from various plant bodies are sown on MS agar medium supplemented with appropriate antibiotics. Arabidopsis thaliana grown on this medium are transferred to pots and grown there. As a result, seeds of a transgenic plant into which DREB gene is introduced can be obtained.

Generally, a transgene is located on the genome of the host plant. However, due to the difference in the locations on the genome, the expression of the transgene varies among transformants, presenting a

phenomenon called position effect. Those transformants in which the transgene is expressed more highly can be selected by assaying mRNA levels in transformants by Northern blot analysis using a DNA fragment from the transgene as a probe.

The confirmation that the gene of interest is integrated in the transgenic plant of the invention and in the subsequent generation thereof can be made by extracting DNA from cells and tissues of those plants by conventional methods and detecting the transgene by PCR or Southern analysis known in the art.

3-3. Analysis of Expression Levels and Expression Sites of DREB Gene in Plant Tissues

Expression levels and expression sites of DREB gene in a transgenic plant into which the gene is introduced can be analysed by extracting RNA from cells and tissues of the plant by conventional methods and detecting the mRNA of DREB gene by RT-PCR or Northern blot analysis known in the art. Alternatively, DREB protein may be analysed directly by Western blotting or the like using an antibody raised against the protein.

3-4. Changes in mRNA Levels of Various Genes in a Transgenic Plant in to which DREB Gene is Introduced

It is possible to identify by Northern blot analysis those genes whose expression levels are believed to have been changed as a result of the action of DREB protein in a transgenic plant into which DREB gene is introduced. Northern blotting can assay those genes by comparing their mRNA levels in the transgenic plant into which DREB gene is introduced and in plants into which the gene is not

introduced.

For example, plants grown on GM agar medium or the like are given dehydration and/or low temperature stress for a specific period of time (e.g. 1 to 2 weeks). Dehydration stress may be given by pulling out the plant from the agar medium and drying it on a filter paper for 10 min to 24 hr. Low temperature stress may be given by retaining the plant at 15 to -4 ℃ for 10 min to 24 hr. Total RNA is prepared from control plants which did not receive any stress and plants which received dehydration and low temperature stresses. The resultant total RNA is subjected to electrophoresis. Then, genes expressing are assayed by Northern blot analysis or RT-PCR.

3-5. Evaluation of the Tolerance to Environmental Stresses of the Tra nsgenic Plant

The tolerance to environmental stresses of the transgenic plant into which DREB gene is introduced can be evaluated by setting the plant in a pot containing a soil comprising vermiculite, perlite and the like exposing the plant to various stresses such as dehydration, low temperature and freezing, and examining the survival of the plant. For example, tolerance to dehydration stress can be evaluated by leaving the plant without giving water for 2 to 4 weeks and then examining the survival. Tolerance to freezing stress can be evaluated by leaving the plant at -6 to -10 ℃ for 5 to 10 days, growing it at 20 to 25 ℃ for 5 to 10 days and then examining its survival ratio.

## PREFERRED EMBODIMENTS OF THE INVENTION

Hereinbelow, the present invention will be described more

specifically with reference to the following Examples. However, the technical scope of the present invention is not limited to these Examples.

EXAMPLE 1

Cloning of DREB1A Gene and DREB2A Gene

(1) Cultivation of Arabidopsis thaliana Plant

Arabidopsis thaliana seeds obtained from LEHLE SEEDS were sterilized in a solution containing 1% sodium hypochlorite and 0.02% Triton X-100 for 15 min. After rinsing with sterilized water, 40-120 seeds were sown on GM agar medium [4.6 g/L mixed salts for Murashige-Skoog medium (Nihon Pharmaceutical Co., Ltd.), 0.5 g/L MES, 30 g/L sucrose, 8 g/L agar, pH 5.7] and cultured at 22 ℃ under conditions of 16 hr light (about 1000 lux) 8 hr dark, to thereby obtain plant.

(2) Preparation of Poly(A)$^+$ RNA

The plant bodies obtained in (1) above were subjected to low temperature treatment at 4 ℃ for 24 hr, and then total RNA was prepared from them by the glyoxal method. Briefly, 3 g of Arabidopsis thaliana plant frozen in liquid nitrogen was suspended in 100 ml of 5.5 M GTC solution (5.5 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sodium N-lauroyl sarcosinate) and solubilized quickly with a homogenizer. This homogenate was sucked into and extruded from a syringe provided with a 18-G needle repeatedly more than 10 times to thereby disrupt the DNA. Then, the homogenate was centrifuged at 4℃ at 12,000xg for 15 min to precipitate and remove the cell debris.

The resultant supernatant was overlayered on 17 ml of CsTFA solution [a solution obtained by mixing cesium trifluoroacetate

(Pharmacia), 0.25 M EDTA and sterilized water to give D=1.51] placed in an autoclaved centrifuge tube, and then ultracentrifuged in Beckmann SW28 Rotor at 15℃ at 25,000 rpm for 24 hr to precipitate RNA.

The resultant RNA was dissolved in 600 μl of 4 M GTC solution (obtained by diluting the above-described 5.5 M GTC solution with sterilized water to give a GTC concentration of 4 M) and precipitated with ethanol to thereby obtain total RNA of interest.

The resultant total RNA was dissolved in 2 ml of TE/NaCl solution (1:1 mixture of TE and 1 M NaCl) and passed through an oligo-dT cellulose column [prepared by packing a Bio-Rad Econocolumn (0.6 cm in diameter) with oligo-dT cellulose (type 3) (Collaborative Research) to a height of 1.5 cm] equilibrated with TE/NaCl in advance. The solution passed through the column was fed to the column again. Subsequently, the column was washed with about 8 ml of TE/NaCl. TE was added thereto to elute and purify poly(A)$^+$ RNA. The amount of the thus obtained RNA was determined with a UV spectroscope.

(3) Synthesis of a cDNA Library

Double-stranded cDNA was synthesized with a cDNA synthesis kit (Stratagene) using 5 μg of the poly(A)$^+$ RNA obtained in (2) above. Then, the double-stranded cDNA was ligated to pAD-GAL4 plasmid (Stratagene) to thereby synthesize a cDNA library. Briefly, at first, single-stranded cDNA was synthesized in the following reaction solution according to the protocol attached to the kit.

| | |
|---|---|
| Poly(A)$^+$ RNA | 5 $\mu$l (5 $\mu$g) |
| 10x 1st Strand synthesis buffer | 5 $\mu$l |
| DEPC-treated water | 34 $\mu$l |
| 40 U/$\mu$l Ribonuclease inhibitor | 1 $\mu$l |
| Nucleotide mix for 1st strand | 3 $\mu$l |
| 1.4 $\mu$g/$\mu$l Linker primer | 2 $\mu$l |
| Total | 50 $\mu$l |

To the above solution, 1.5 $\mu$l (50 U/$\mu$l) of reverse transcriptase was added and incubated at 37 ℃ for 1 hr to thereby synthesize single-stranded cDNA. To the resultant reaction solution containing single-stranded cDNA, the following reagents were added in the indicated order.

| | |
|---|---|
| Reaction solution containing single-stranded cDNA | 45 $\mu$l |
| 10x 2nd Strand synthesis buffer | 20 $\mu$l |
| NTP mix for 2nd strand | 6 $\mu$l |
| 1.5 U/$\mu$l RNase H | 2$\mu$l |
| 9 U/$\mu$l DNA polymerase I | 11$\mu$l |
| DEPC-treated water | 116 $\mu$l |
| Total | 200 $\mu$l |

The resultant reaction solution was incubated at 16℃ for 2.5 hr to thereby synthesize double-stranded cDNA.

The resultant double-stranded cDNA was blunt-ended by incubating it with 5 units of Pfu DNA polymerase at 72 ℃ for 30 min. Subsequently, the resultant cDNA was subjected to phenol/chloroform

extraction and ethanol precipitation. To the resultant pellet, 9 μl of EcoRI-NotI-BamHI adaptor (Takara), 1 μl of 10x ligase buffer, 1 μl of ATP and 1 μl of T4 DNA ligase (4 U/μl) were added and incubated at 4℃ for 2 days to thereby add the adaptor to the double-stranded cDNA.

Subsequently, the cDNA having an EcoRI restriction enzyme site at both ends was ligated to the EcoRI site downstream of the GAL4 activation domain of pAD-GAL4 plasmid (Stratagene) (a cloning vector) with T4 DNA ligase to thereby synthesize a cDNA library.

(4) Preparation of Genomic DNA

Genomic DNA was prepared from the plant obtained in (1) above according to the method described by Maniatis, T. et al. [Molecular Cloning: A Laboratory Manual, pp. 187-198, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1982)]. Briefly, 2,000 ml of disruption buffer [0.35 M sucrose, 1 M Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl] was added to 50 g of Arabidopsis thaliana plant. The mixture was disrupted in a whirling blender for 1 min 3 times to homogenize the plant bodies.

The disrupted material was filtered to remove the cell residue. The filtrate was dispersed into centrifuge tubes and centrifuged in a swing rotor at 3,000xg at 4 ℃ for 10 min at a low speed. The resultant supernatant was discarded. The precipitate was suspended in 30 ml of ice-cooled disruption buffer and then re-centrifuged at a low speed. The same procedures were repeated 3 times until the green precipitate turned into white.

The resultant white precipitate was suspended in 10 ml of ice-cooled TE. To this suspension, 10 ml of lysis solution (0.2 M Tris-

HCl (pH 8.0), 50 mM EDTA, 2% sodium N-lauroyl sarcosinate) was added. Then, 0.1 ml of proteinase K (10 mg/ml) was added thereto to digest nuclei. The resultant digest was subjected to phenol treatment and ethanol precipitation. The DNA fiber obtained by the precipitation was recovered by centrifugation at 3,000xg for 5 min and dissolved in 1 ml of TE to thereby obtain genomic DNA.

(5) Construction of a Host Yeast for Use in Yeast One Hybrid Screening

For the cloning of a gene encoding the transcription factor (DRE-binding protein) to be used in the invention, a host was constructed (Fig. 1). This host for cloning comprises two plasmids, one containing 4 cassettes of DRE motif-containing DNA upstream of HIS3 reporter gene and the other containing 4 cassettes of DRE motif-containing DNA upstream of lacZ reporter gene. Briefly, first, the promoter region of rd29A gene (the region from -215 to -145 based on the translation initiation point of rd29A gene) comprising DRE sequence to which the transcription factor to be used in the invention binds to was amplified by PCR. As a sense primer, 5'-aagcttaagcttacatcagtttgaaagaaa-3' (SEQ ID NO: 11) was synthesized. As an antisense primer, 5'-aagcttaagcttgcttttttggaactcatgtc-3' (SEQ ID NO: 12) was synthesized. To these primers, a HindIII restriction site was introduced to their 5'end so that PCR fragments can be ligated to a vector easily after amplification. These primers were synthesized chemically with a fully automated DNA synthesizer (Perkin-Elmer). A PCR was performed using these primers and the genomic DNA from (4) above as a template. The composition of the PCR reaction solution was as follows.

| | |
|---|---|
| Genomic DNA solution | 5 μl (100 ng) |
| Sterilized water | 37 μl |
| 10x PCR buffer [1.2 M Tris-HCl (pH 8.0), 100 mM KCl, 60 mM $(NH_4)_2SO_4$, 1% Triton X-100, 0.1 mg/ml BSA] | 5 μl |
| 50 pmol/μl Sense primer | 1μl (50 pmol) |
| 50 pmol/μl Antisense primer | 1μl (50 pmol) |
| KOD DNA polymerase (KOD-101, TOYOBO) | 1μl (2.5 U) |
| Total | 50 μl |

After the above reaction solution was mixed thoroughly, 50 μl of mineral oil was overlayered on it. The PCR was performed 25 cycles, one cycle consisting of thermal denaturation at 98℃ for 15 sec, annealing at 65℃ for 2 sec and extension at 74℃ for 30 sec. After completion of the reaction, 50 μl of chloroform was added to the reaction solution, and then the resultant mixture was centrifuged at 4 ℃ at 15,000 rpm for 15 min. The resultant upper layer was recovered into a fresh microtube, to which 100 μl of ethanol was added and mixed well. The mixture was centrifuged at 4℃ at 15,000 rpm for 15 min to pellet the PCR product.

The resultant PCR product was digested with HindIII and then ligated to the HindIII site of vector pSK to yield a recombinant plasmid. This plasmid was transformed into E. coli. From the transformant, plasmid DNA was prepared to determine the nucleotide sequence. By these procedures, a transformant comprising pSK with a DNA fragment containing 4 cassettes of DRE connected in the same direction was selected.

The DNA fragment containing 4 cassettes of DRE was cut out from pSK plasmid using EcoRI and HincII, and then ligated to the EcoRI-MluI site upstream of the HIS3 minimum promoter of a yeast expression vector pHISi-1 (Clontech). Likewise, the DRE-containing DNA fragment was cut out from pSK plasmid using EcoRI and HincII, and then ligated to the EcoRI-SalI site upstream of the lacZ minimum promoter of a yeast expression vector pLacZi (Clontech). The resultant two plasmids were transformed into Saccharomyces cerevisiae YM4271 (MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-903) (Clontech) to thereby yield a host yeast to be used in yeast one hybrid screening (Fig. 1).

(6) Cloning of DREB1A Gene and DREB2A Gene

The host yeast prepared in (5) above was transformed with the cDNA library prepared in (3) above. The resultant yeast transformants ($1.2 \times 10^6$) were cultured and screened as described previously. As a result, two positive clones were obtained. The cDNAs of these clones were cut out from pAD-GAL4 plasmid using EcoRI and then ligated to the EcoRI site of pSK plasmid to thereby obtain recombinant plasmids pSKDREB1A and pSKDREB2A.

(7) Determination of the Nucleotide Sequences

The entire nucleotide sequences for the cDNAs were determined using plasmids pSKDREB1A and pSKDREB2A. These plasmids were prepared with an automated plasmid preparation apparatus Model PI-100 (Kurabo). For the sequencing reaction, a reaction robot CATALYST 800 (Perkin Elmer) was used. For the DNA sequencing, Perkin Elmer Sequencer Model

373A was used. As a result, it was found that the cDNA from plasmid pSKDREB1A consists of 933 bp (SEQ ID NO: 1) and that only one open reading frame exists therein which encodes a protein consisting of 216 amino acid residues with a presumed molecular weight of about 24.2 kDa (SEQ ID NO: 2). On the other hand, it was found that the cDNA from plasmid pSKDREB2A consists of 1437 bp (SEQ ID NO: 3) and that only one open reading frame exists therein which encodes a protein consisting of 335 amino acid residues with a presumed molecular weight of about 37.7 kDa (SEQ ID NO: 4).

(8) Isolation of Genes Encoding Homologues to DREB1A or DREB2A Protein

Genes encoding homologues to the protein encoded by DREB1A or DREB2A gene obtained in (6) above were isolated. Briefly, genes encoding such homologues were isolated from Arabidopsis thaliana λ gt11 cDNA library using as a probe a double-stranded cDNA fragment comprising DREB1A or DREB2A gene according to the method described by Sambrook, J. et al., Molecular Cloning: A Laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory Press, NY (1989). As genes encoding homologues to DREB1A protein, DREB1B gene and DREB1C gene were obtained; as a gene encoding a homologue to DREB2A protein, DREB2B gene was obtained. As a result of DNA sequencing, it was found that DREB1B gene (SEQ ID NO: 5) was identical with the gene called CBF1 [Stockinger, E.J. et al., Proc. Natl. Acad. Sci. USA 94:1035-1040 (1997)], but DREB1C gene (SEQ ID NO: 7) and DREB2B gene (SEQ ID NO: 9) were found to be novel.

From the analysis of the open reading frame of DREB1C gene, it was found that the gene product encoded by this gene is a protein

consisting of 216 amino acid residues with a molecular weight of about 24.3 kDa (SEQ ID NO: 8). Also, it was found that the gene product encoded by DREB2B gene is a protein consisting of 330 amino acid residues with a molecular weight of about 37.1 kDa (SEQ ID NO: 10).

## EXAMPLE 2

### Analysis of the DRE-Binding Ability of DREB1A and DREB2A Proteins

The ability of DREB1A and DREB2A proteins to bind to DRE was analyzed by preparing a fusion protein composed of glutathione-S-transferase (GST) and DREB1A or DREB2A protein using E. coli and then performing a gel shift assay. Briefly, the 429 bp DNA fragment from position 119 to position 547 of the nucleotide sequence of DREB1A cDNA or the 500 bp DNA fragment from position 167 to position 666 of the nucleotide sequence of DREB2A cDNA was amplified by PCR. Then, the amplified fragment was ligated to the EcoRI-SalI site of plasmid pGEX-4T-1 (Pharmacia). After the introduction of this plasmid into E. coli JM109, the resultant transformant was cultured in 200 ml of 2x YT medium (Molecular Cloning, (1982) Cold Spring Harbor Laboratory Press). To this culture, 1 mM isopropyl β-D-thiogalactoside which activates the promoter in plasmid pGEX-4T-1 was added to thereby induce the synthesis of a fusion protein of DREB1A (or DREB2A) and GST.

E. coli in which the fusion protein had been induced was suspended in 13 ml of buffer (10 mM Tris-HCl, 0.1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride). Then, 1% Triton X-100 and 1 mM EDTA were added thereto. After the cells were disrupted by sonication, the disrupted material was centrifuged at 22,000xg for 20 min. Then, the

fusion protein of DREB1A (or DREB2A) and GST was purified by affinity chromatography using glutathione-Sepharose (Pharmacia) as a carrier. The resultant fusion protein was incubated with the DRE-containing 71 bp DNA fragment probe prepared by PCR and radiolabelled with $^{32}P$ at room temperature for 20 min. This mixture was electrophoresed using 6% acryl amide gel containing 0.25xTris-borate-EDTA at 100 V for 2 hr. Figs. 2A and 2B show the results of autoradiogram on the gel after the electrophoresis. As is clear from this Figure, a band which migrated behind was detected when the fusion protein was incubated with the DRE-containing 71 bp DNA fragment probe (SEQ ID NO: 18). When a DNA fragment containing a varied DRE sequence (SEQ ID NO: 19, 20 or 21) was used, such a band was not detected. On the other hand, when a DNA fragment which was partly varied outside of DRE sequence (SEQ ID NO: 22 or 23) was used as a probe, a behind band was detected. Thus, it was shown that DREB1A or DREB2A protein specifically bound to DRE sequence.

## EXAMPLE 3

### Analysis of the Ability of DREB1A and DREB2A Proteins to Activate the Transcription of Genes Located Downstream of DRE

In order to examine whether DREB1A and DREB2A proteins are able to trans-activate DRE-dependent transcription in plant cells, a trans-activation experiment was conducted using a protoplast system prepared from Arabidopsis thaliana leaves. Briefly, the cDNA of DREB1A or DREB2A was ligated to a pBI221 plasmid containing CaMV35S promoter to thereby construct an effector plasmid. On the other hand, 3 cassettes of the DRE-containing 71 bp DNA region were connected tandemly to prepare a DNA fragment, which was then ligated upstream to

the minimum TATA promoter located upstream of β-glucuronidase (GUS) gene in a plasmid derived from pBI221 plasmid to construct a reporter plasmid. Subsequently, these two plasmids were introduced into protoplasts from Arabidopsis thaliana and then GUS activity was determined. When DREB1A or DREB2A protein was expressed simultaneously, GUS activity increased. This shows that DREB1A and DREB2A proteins are transcription factors which activate transcription through DRE sequence (Fig. 3B).

EXAMPLE 4

Creation of a Transgenic Plant Containing a Gene in which a DNA Encoding DREB1A Protein is Ligated Downstream of CaMV35S Promoter

(1) Construction of a Plant Plasmid

Plasmid pSKDREB1A (10 μg) obtained as described above was digested with EcoRV (20 U) and SmaI (20 U) in a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 100 mM NaCl at 37℃ for 2 hr to thereby obtain a DNA fragment of about 0.9 kb containing DREB1A gene. On the other hand, plasmid pBI2113Not (10 μg) containing promoter DNA was digested with SmaI in a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT and 100 mM NaCl at 37 ℃ for 2 hr. The 0.9 kb DNA fragment containing DREB1A gene and the digested pBI2113Not were treated with T4 DNA ligase (2 U) in a buffer [66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT, 0.1 mM ATP] at 15 ℃ for 16 hr for ligation. The ligated DNA was transformed into E. coli JM109. The transformant was cultured, and plasmid pBI35S:DREB1A was obtained from the culture. Then, the nucleotide sequence was determined, and those plasmids in which

DREB1A gene was ligated in the sense direction were selected. Plasmid pBI2113Not mentioned above is a plasmid prepared by digesting pBI2113 [Plant Cell Physiology 37:49-59 (1996)] with SmaI and SacI to remove the coding region of GUS gene and ligating a SmaI-NotI-SacI polylinker to the resultant plasmid.

(2) Preparation of a Zygote Agrobacterium Containing the Plant Plasmid pBI35S:DREB1A

E. coli DH5α containing the plant plasmid pBI35S:DREB1A prepared in (1) above, E. coli HB101 containing helper plasmid pRK2013 and Agrobacterium C58 were cultured in mixture on LB agar medium at 28 ℃ for 24 hr. Grown colonies were scraped off and suspended in 1 ml of LB medium. This suspension (10 ml) was plated on LB agar medium containing 100 μg/ml rifampicin and 20 μg/ml kanamycin and cultured at 28 ℃ for 2 days to thereby obtain a zygote Agrobacterium C58 (pBI35S:DREB1A).

(3) Gene Transfer into Arabidopsis thaliana by Agrobacterium Infection

The resultant zygote Agrobacterium was cultured in 10 ml of LB medium containing 100 μg/ml rifampicin and 20 μg/ml kanamycin at 28℃ for 24 hr. Further, this culture fluid was added to 500 ml of LB medium and cultured for another 24 hr. The resultant culture fluid was centrifuged to remove the medium, and the cell pellet was suspended in 250 ml of LB medium.

On the other hand, 4 to 5 Arabidopsis thaliana plant bodies were grown in 9 cm pots containing soil composed of vermiculite and perlite (50:50) for 6 weeks. Then, the plant body was directly dipped

in the LB culture fluid of the Agrobacterium containing plasmid pBI35S:DREB1A and placed in a desiccator, which was sucked with a vacuum pump to reduce the pressure to 650 mmHg and then left for 10 min. Subsequently, the plant pot was transferred to a tray and covered with a wrap to maintain the humidity. The next day, the wrap was removed. Thereafter, the plant was grown uncovered to thereby obtain seeds. After sterilization in an aqueous solution of sodium hypochlorite, the seeds were sown on an agar medium for selection (MS medium supplemented with 100 $\mu$ g/ml vancomycin and 30 $\mu$ g/ml kanamycin). Arabidopsis thaliana seedlings grown on this medium were transplanted to pots and grown there to obtain seeds of the transformed plant.

(4) Identification of Genes Whose Expression Has Been Altered by the Transgene and the Transcription Factor Encoded by the Transgene

mRNA levels of those genes whose expression is considered to have been altered by the transgene DREB1A and the transcription factor encoded by this gene in the transformed plant were examined by Northern blot analysis. As a probe, a fragment of DREB1A, rd29A, kin1, cor6.6, cor15a, rd17, erd10, P5CS, erd1, rd22 or rd29B gene was used. In this Northern blot analysis, transformed and wild type Arabidopsis thaliana plants were used for comparing the expression of the above genes. Two grams each of plant bodies grown on GM agar medium for 3 weeks were exposed to dehydration stress and low temperature stress separately. Dehydration stress was given by pulling out the plant from the agar medium and drying it on a filter paper for 5 hr. Low temperature stress was given by retaining the plant at 4℃ for 5 hr.

Total RNA was prepared separately from control plants which were given no stress, plants which were given dehydration stress and plants which were given low temperature stress. The resultant total RNA was subjected to electrophoresis. Then, expressing genes were assayed by Northern blot analysis. Generally, a transgene is located on the genome of a transformed plant in a similar manner. However, due to the difference in the locations on the genome, the expression of the transgene varies among transformants; this is a phenomenon called position effect. By assaying transformants by Northern blotting with a DNA fragment from the transgene as a probe, those transformants in which the transgene was expressed more highly were selected. Also, by using as a probe a DNA fragment of the above genes which are possibly involved in stress tolerance, those genes which exhibited changes in mRNA levels when DREB1A gene was introduced were identified (Fig. 5).

(5) Expression of Tolerance to Dehydration/Freezing Stress

Dehydration/freezing tolerance was investigated on Arabidopsis thaliana transformants which had been grown in 9 cm pots containing soil composed of vermiculite and perlite (50:50) for 3 weeks. As a control, Arabidopsis thaliana transformed with pBI121 not containing DREB1A gene was used. As to dehydration tolerance, water supply was stopped for 2 weeks and then plant survival was examined. As to freezing tolerance, the plant was maintained at -6℃ for 2 days and then grown at 22℃ for 5 days. Thereafter, its survival ratio was examined.

As a result, all the control plants were withered but the transgenic plants into which DREB1A gene was introduced exhibited a

high survival ratio (Fig. 6). However, inhibition of growth and dwarfing were observed in these transgenic plants.

## EXAMPLE 5

### Creation of a Transgenic Plant Containing a Gene in which a DNA Encoding DREB1A Protein is Ligated Downstream of rd29A Gene Promoter

(1) Construction of pBI29APNot Vector Containing rd29A Gene Promoter

An rd29A promoter region (from -861 to +63 based on the translation initiation point of rd29A gene) with HindIII site added to both ends was prepared by PCR under the same conditions as described in (4) in Example 2 using the following primers: 5'-aagcttaagcttgccatagatgcaattcaatc-3' (SEQ ID NO:13) and 5'-aagcttaagcttttccaaagatttttttctttccaa-3' (SEQ ID NO: 14). The resultant PCR fragment was digested with HindIII and inserted into the HindIII site of a plant binary vector pBI101 (Clontech, Palo Alto, CA, USA). $\beta$-glucuronidase gene (GUS) encoded in pBI101 was cut out with SmaI and SacI. Then, the resultant plasmid was ligated with SmaI-NotI-SacI polylinker. This plasmid was introduced into E. coli DH5a to prepare plasmid pBI29APNot.

(2) Construction of Plant Plasmid pBI29AP:DREB1A Using rd29A Gene Promoter

DREB1A gene was amplified by PCR using pSKDREB1A obtained in Example 1 as a template. Briefly, 5'-ggatccggatccatgaactcattttctgct-3' (SEQ ID NO: 15) was synthesized as a sense primer and 5'-ggatccggatccttaataactccataacgata-3' (SEQ ID NO: 16) as an

antisense primer. BamHI site was introduced at 5' end of both primers so that the PCR fragment amplified can be ligated to the vector easily. The resultant PCR product was subjected to electrophoresis on 1% agarose gel. A band around 900-1000 bp was cut out from the gel. This gel fragment was placed in a fresh microtube, which was retained at 67 ℃ for 10 min to dissolve the gel. An equal volume of TE was added to the dissolved gel, mixed well and extracted with phenol. The resultant extract was centrifuged at 1,600xg for 3 min. Then, the aqueous layer was subjected to phenol extraction and phenol/chloroform extraction. To the resultant aqueous layer, cold ethanol was added to precipitate the PCR product.

The resultant PCR product (10 $\mu$g) was dissolved in 30 $\mu$l of TE and digested with BamHI (20 U). After heating at 70℃ for 1 hr to deactivate BamHI, the digest was subjected to phenol extraction and ethanol precipitation to recover a DNA fragment containing DREB1A gene. Subsequently, this DNA fragment was ligated to the BamHI site of vector pBI29APNot. This recombinant plasmid was transformed into E. coli (DH5 $\alpha$), and the transformant was selected by kanamycin resistance. The selected transformant was cultured in LB medium. Then the plasmid pBI29AP:DREB1A was extracted and purified from the transformant (Fig. 7).

(3) Preparation of a Zygote Agrobacterium Containing Plant Plasmid pBI29AP:DREB1A

Using the recombinant plasmid pBI29AP:DREB1A obtained in (2) above, a zygote Agrobacterium containing plant plasmid pBI29AP:DREB1A was prepared in the same manner as in (2) in Example 5.

(4) Gene Transfer into Arabidopsis thaliana by Agrobacterium Infection

Using the zygote Agrobacterium obtained in (3) above, plant plasmid pBI29AP:DREB1A was introduced into Arabidopsis thaliana in the same manner as in (3) in Example 5.

(5) Observation of the Growth and Dehydration/Freezing/Salt Stress Tolerance of the Transformant

The transgenic Arabidopsis thaliana obtained in (4) above containing a plasmid in which DREB1A gene is ligated downstream of rd29A gene promoter, the transgenic Arabidopsis thaliana obtained in Example 5 containing a plasmid in which DREB1A gene is ligated downstream of CaMV35S gene promoter, and non-transformed Arabidopsis thaliana as a control were cultured under the same conditions. Then, their growth and survival ratios after the loading of dehydration, freezing or salt stress were examined. Briefly, each plant was planted in a 9 cm pot containing soil composed of vermiculite and perlite (50:50) and cultured outside. Figs. 8 and 9 present photographs showing the growth of plants on day 35 (Fig. 8A and Fig. 9A) and on day 65 (Fig. 8B and Fig. 9B) of the cultivation. In the pBI35S:DREB1A-introduced transgenic plant, a remarkable inhibition of growth was observed though there was some difference in the degree of growth among plants (Fig. 8A and Fig. 8B). In contrast, almost no inhibition of growth was observed in the pBI29AP:DREB1A-introduced transgenic plant (Fig. 9A and Fig. 9B).

Subsequently, their tolerance to stresses was examined. As to dehydration tolerance, water supply was stopped for 2 weeks and then plant survival was examined. As to freezing tolerance, plants were

maintained at -6 ℃ for 2 days and then grown at 22℃ for 5 days. Thereafter, their survival ratios were examined. As to salt tolerance, plants were dipped in 600 mM NaCl for 2 hrs, then transferred to pots and grown there for 3 weeks. Thereafter, plant survival was examined. As a result, as shown in Fig. 10 and Tables 1 to 3, the control plants given dehydration or freezing stress were all withered. Only few control plants survived after the loading of salt stress. In the pBI35S:DREB1A-introduced transformant, the survival ratio varied among plants; those plants with higher expression of the introduced DREB1A gene exhibited higher tolerance. In contrast, in the pBI29AP:DREB1A-introduced transformant, the tolerance was almost equal among 43 plants analyzed. This transformant exhibited higher survival ratios than the pBI35S:DREB1A-introduced transformant. Thus, it was found that the transgenic plant created by the invention has high levels of tolerance to dehydration, freezing and salt, and yet exhibits good growth.

Table 1.
Survival Ratio of Transgenic Plants after the Loading of Freezing Stress

| | No. of Individuals Survived | Total No. of Individuals | Survival Ratio (%) |
|---|---|---|---|
| rd29A:DREB1A | 143 | 144 | 99.3 |
| 35S:DREB1Ab | 47 | 56 | 83.9 |
| 35S:DREB1Ac | 15 | 42 | 35.7 |
| Wild type | 0 | 55 | 0.0 |

Table 2.
Survival Ratio of Transgenic Plants after the Loading of Dehydration Stress

| | No. of Individuals Survived | Total No. of Individuals | Survival Ratio(%) |
|---|---|---|---|
| rd29A:DREB1A | 52 | 80 | 65.0 |
| 35S:DREB1Ab | 15 | 35 | 42.9 |
| 35S:DREB1Ac | 6 | 28 | 21.4 |
| Wild type | 0 | 25 | 0.0 |

Table 3.
Survival Ratio of Transgenic Plants after the Loading of Salt Stress

| | No. of Individuals Survived | Total No. of Individuals | Survival Ratio(%) |
|---|---|---|---|
| rd29A:DREB1A | 119 | 149 | 79.9 |
| 35S:DREB1Ab | 4 | 24 | 16.7 |
| Wild type | 4 | 29 | 13.8 |

All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

## EFFECT OF THE INVENTION

According to the present invention, there is provided a transgenic plant containing a gene in which a DNA coding for a protein that binds to a stress responsive element and regulates the transcription of genes located downstream of the element is ligated downstream of a stress responsive promoter, the transgenic plant having improved tolerance to environmental stresses (such as dehydration, low temperature and salt) and being free from dwarfing.